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14. ABSTRACT

Betulinic acid (BA) is a triterpenoid anticancer agent, and treatment of epidermal growth factor 2 (EGFR2, HER2/ErbB2)-overexpressing BT474 and MDA-MB-453 cells with 1-10 μM BA inhibited cell growth and induced apoptosis. BA also induced proteasome-independent downregulation of specificity protein (Sp) transcription factors Sp1, Sp3, Sp4 and survivin, a Sp-regulated gene, and decreased expression of ErbB2, ErbB2-regulated kinases and YY1, a transcription factor that regulates ErbB2 expression. Knockdown of Sp1, Sp3, Sp4 and their combination by RNA interference was accompanied by decreased expression of ErbB2, YY1 and luciferase activity in cells transfected with a construct containing the GC-rich YY1 promoter linked to a luciferase reporter gene. BA-dependent repression of Sp1, Sp3, Sp4 and Sp regulated genes was due, in part, to induction of the Sp repressor ZBTB10 and downregulation of microRNA-27a (miR-27a) which constitutively inhibits ZBTB10 expression. The effects of BA on the miR-27a:zBTB10-Sp transcription factor axis were inhibited in cells cotreated with the cannabinoid 1 (CB1) and CB2 receptor antagonists AM251 and AM630, respectively. However, in vitro binding studies with ≤10 μM BA and a radiolabled cannabinoid did not indicate competitive binding of BA to the CB1 and CB2 receptors, suggesting a possible role for other CB-like G protein-coupled receptors.

15. SUBJECT TERMS

betulinic acid, Sp transcription factors, microRNA-27a, ZBTB10, cannabinoid receptors

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INTRODUCTION

Betulinic acid (BA) is relatively non-toxic in rodent studies and highly effective against melanoma in both *in vivo* and *in vitro* assays (1). Subsequent research in several laboratories indicates that BA inhibits growth of multiple tumor types including breast cancer (2, 3). Studies in this laboratory show that BA inhibits prostate cancer cell and tumor growth in a xenograft model, and one of the underlying mechanisms of action is due to BA-induced proteasome-dependent degradation of specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4 (4). These proteins are highly expressed in several different cancer cell lines and tumors (4-9), whereas Sp1 levels in non-tumor tissue of rodents and humans is relatively low and decreases with age (10, 11). In this study supported by the DOD grant, we have used BA as a model to investigate the effect of Sp1, Sp3 and Sp4 downregulation on BT474 and MDA-MD-453 breast cancer cells that express the oncogene EGFR2 (ErbB2, HER2). The proposed research has focused on the role of BA-induced Sp downregulation on cell and tumor growth, ErbB2 expression and the overall mechanisms associated with the anticancer activity of BA.

BODY

1. BA inhibits growth, induces apoptosis and downregulates Sp1, Sp3 and Sp4 in BT474 and MDA-MB-453 cells

BT474 and MDA-MB-453 cells overexpress ErbB2 and these cell lines are widely used as models for understanding the molecular mechanisms associated with drugs that target cancer cells expressing high levels of ErbB2. BA inhibited proliferation of both BT474 and MDA-MB-453 cells and the overall decrease in cell number was both concentration (1, 5 and 10 μM)- and time (2 or 4 days)-dependent, and MDA-MB-453 cells were less responsive to BA than BT474 cells (Figs. 1A and 1B). The growth inhibitory effects of BA were also accompanied by induction of cleaved PARP, a marker of apoptosis, and decreased expression of survivin, an inhibitor of apoptosis (Fig. 1C). Induction of apoptosis by BA in BT474 and MDA-MB-453 was also confirmed in a TUNEL assay in which BA increased TUNEL staining in both cell lines (Fig. 1D).

The growth inhibitory effects of BA in LNCaP prostate cancer cells have been linked, in part, to activation of proteasome-dependent degradation of Sp1, Sp3 and Sp4 proteins (4) and results illustrated in Figures 2A show that, after treatment of BT474 and MDA-MB-453 cells with 10 μM BA for 48 hr, there was a decrease in expression of Sp1, Sp3, Sp4 and survivin (an Sp-regulated gene) proteins in both cell lines. The proteasome inhibitor MG132 alone was cytotoxic to BT474 and MDA-MB-453 cells and MG132 alone decreased Sp proteins, whereas lactacystin was not toxic and did not affect Sp protein expression (data not shown). Treatment of BT474 or MDA-MB-453 cells with 10 μM BA plus 1 μM lactacystin for 48 hr resulted in decreased expression of Sp1, Sp3 Sp4 and survivin proteins that was not blocked after cotreatment with lactacystin, suggesting that BA-mediated downregulation of Sp proteins was proteasome-independent. Therefore, the effects of 10 μM BA on Sp1, Sp3 and Sp4 mRNA levels were investigated and, after treatment of BT474 and MDA-MB-453 (Fig. 2C) for 16 hr, there was a significant decrease in Sp1, Sp3 and Sp4 mRNA levels, suggesting that the mechanism of action of BA involved transcriptional repression.

2. BA downregulates ErbB2 and ErbB2-regulated genes

Since ErbB2 plays a major role in the proliferation of BT474 and MDA-MB-453 cells, the effects of BA alone and in combination with lactacystin on ErbB2, MAPK, Akt and their phosphorylated analogs were investigated. BA alone decreased ErbB2, p-ErbB2, and downstream kinases MAPK, p-MAPK, Akt and p-Akt expression (Fig. 2C) and these effects were not reversed after co-incubation with the proteasome inhibitor lactacystin. We also investigated the effects of BA alone and BA-plus lactacystin on expression of YY-1 which is a key upstream regulator of ErbB2 in breast cancer cells overexpressing this oncogene (12). The results showed that BA decreased expression of YY1 in both cell lines in the presence or absence of lactacystin (Fig. 2C). The linkage between Sp transcription factors and ErbB2 could be due to regulation of YY1 by Sp transcription since the YY1 promoter contains multiple GC-rich Sp binding sites (13). We also investigated the effects of BA on YY1 promoter activity. In MDA-MB-453 cells transfected with YY1 p-277 Luc or p-1729 Luc, two luciferase reporter constructs containing the -277bp and -1729bp region from the YY1 promoter, treatment with BA for 24 hr resulted in a dose-dependent decrease in luciferase activity (Fig. 2D).

RNA interference was used to investigate the potential role of Sp proteins in mediating basal expression of YY1 in BT474 and MDA-MB-453 cells. Figures 3A and 3B illustrate the effect of siRNAs against Sp1 (iSp1), Sp3 (iSp3), Sp4 (iSp4) and their combination (iSp1/3/4) on expression of Sp proteins, YY1 and ErbB2. Transfection of BT474 cells with two siRNAs for Sp1, iSp3, iSp4 and iSp1/3/4 resulted in specific knockdown of the target Sp proteins and Sp1, iSp3, iSp4 and iSp1/3/4 and also decreased expression of YY1 and ErbB2 proteins. In a second set of experiments, the oligonucleotides were also transfected in MDA-MB-453 cells (Fig. 3B). The siRNAs for Sp1 and Sp4 proteins were highly specific; however, iSp3 decreased expression Sp3 and Sp4 proteins. iSp1, iSp4 and iSp1/3/4 decreased levels of both YY1 and ErbB2, whereas Sp3 knockdown had minimal effects on either YY1 or ErbB2 proteins. Previous RNA interference studies showed that knockdown of YY1 also decreased expression of ErbB2 and in this study, we also observed that YY1 knockdown decreased ErbB2 in both cell lines (Fig. 3C).

3. Role of cannabinoid receptors

BA-induced downregulation of Sp transcription factors was proteasome-independent (Fig. 2) and these effects were also not reversed by ROS inhibitors (data not shown) as previously reported for CDDO-Me (14). Since preliminary studies in other cancer cell lines show that cannabinoids (CBs) decrease Sp proteins (data not shown), the effects of CB1 and CB2 receptor antagonists AM251 and AM630, respectively, and capsazepine (vanilloid receptor antagonists) on BA-mediated repression of Sp1, Sp3 and Sp4 and survivin were also determined in BT474 and MDA-MB-453 cells (Figs. 4A and 4B). In BT474 and MDA-MB-453 cells, cotreatment of BA with either AM251 or AM630 attenuated the effects of BA downregulation of Sp1, Sp3 and Sp4, whereas capsazepine inhibited the effects of BA only in MDA-MB-453 cells. The effects of AM251, AM630 and capsazepine on BA-mediated downregulation of ErbB2 and ErbB2-regulated kinases were also determined in BT474 (Fig. 4C) and MDA-MB-453 (Fig. 4D) cells. In BT474 cells, the CB1 and CB2 receptor antagonists inhibited BA-mediated downregulation of ErbB2, P-ErbB2, p-MAPK, p-Akt and YY1. Similar results were observed in MDA-MB-453 cells confirming a role for the cannabinoid receptors in mediating the effects of BA on Sp and Sp-regulated genes. Moreover, in MDA-MB-453 cells capsazepine was also active as an inhibitor. Both the CB1 and CB2 receptors are expressed in BT474 and MDA-MB-453 cells (Fig. 4D); however, competitive binding of BA to the CB1 and CB2 receptors using the non-selective radioligand [^{3}H]CB-55,940 showed that BA at concentrations up to 10 μM did not displace the radioligand. Enhanced binding of [^{3}H]CB-

55,940 to both receptors was observed after coincubation with 100 μM BA and since similar effects were observed for both CB receptors, this may be due to non-specific interactions. As a positive control, the synthetic cannabinoid WIN-55,212-2 displaced the radioligand from both the CB1 and CB2 receptors (data not shown). Since BA-mediated inhibition of fatty acid amide hydrolase (FAAH) would increase production of endocannabinoids, we investigated the effects of FAAH knockdown by RNA interference on expression of Sp proteins and ErbB2 (Supplemental Figure 1). The results show that FAAH knockdown does not affect expression of Sp1 or ErbB2, suggesting that the effects of BA are not due to inhibition of FAAH.

Previous studies showed that drug-induced repression of Sp1, Sp3 and Sp4 mRNA levels was due to downregulation of miR-27a and induction of ZBTB10, a transcriptional repressor of Sp1, Sp3 and Sp4 gene expression (6, 8, 14). Treatment of BT474 and MDA-MB-453 cells with 5 or 10 μM BA resulted in significant downregulation of miR-27a in both cell lines and cotreatment with AM251 or AM630 inhibited this response (Fig. 5A) which was most pronounced in BT474 cells. Downregulation of miR-27a in cells treated with BA was accompanied by induction of ZBTB10 mRNA levels in both cell lines and cotreatment with AM251 or AM630 inhibited the induction response (Fig. 5B). A >6-fold induction of ZBTB10 was observed in BT474 cells, whereas ZBTB10 was induced >2.5-fold in MDA-MB-453 cells treated with 5 or 10 μM BA. The effects of antisense-miR-27a (as-miR-27a) and ZBTB10 overexpression on levels of Sp1, Sp3 and Sp4, YY1 and ErbB2 proteins were also determined in BT474 and MDA-MB-453 cells (Figs. 5C and 5D) and both treatments decreased expression of Sp and Sp-regulated gene products. The effects of a miR-27a mimic and as-miR-27a on luciferase activity in BT474 and MDA-MB-453 cells transfected with ZBTB10 (UTR)-luc construct containing a miR-27a binding site resulted in decreased (miR-27a mimic) and increased (as-miR-27a) luciferase activity. In contrast, the mimic or antisense oligonucleotide did not affect luciferase activity in cells transfected with a construct [ZBTB10 (mUTR)-luc] containing a mutation in the miR-27a binding sites, confirming interactions of miR-27a with the target sequence in the 3'-UTR of ZBTB10.

The in vivo effects of BA on tumor growth were also investigated in athymic nude mice bearing BT474 cells as xenografts. BA was administered over a period of 28 days at a dose of 20 mg/kg/d, and tumor volumes and tumor weight were significantly inhibited (Figs. 6A and 6B) and BA decreased expression of Sp1, Sp3 and Sp4 proteins in tumors (Fig. 6C). Figure 6D illustrates that immunostaining of ErbB2 and Sp1 proteins were also decreased in fixed tumor tissue from BA-treated mice compared to control (corn oil) treat animals and these in vivo data complement the results of in vitro studies.

KEY RESEARCH ACCOMPLISHMENTS

- BA decreased expression of Sp1, Sp3, Sp4 and ErbB2 in BT474 and MDA-MB-231 cells.
- BA also decreased expression of YY1 and YY1 regulates ErbB2 expression in these cells.
- YY1 was characterized as another Sp-regulated.
- BA induced Sp downregulation was proteasome-independent and due to miR-27a downregulation and induction of the Sp repressor ZBTB10.
- The upstream targets of BA are the cannabinoid receptors and binding studies are currently being investigated.

REPORTABLE OUTCOMES

- This paper was part of Xinyi Liu's doctoral thesis.
- A manuscript for this work has been prepared and will be submitted within a month.
- An abstract entitled "Betulinic Acid Inhibits BT474 and SKBR3 Breast Cancer Cell Growth by Targeting Sp Proteins and ErbB2" was presented at the annual meeting of the Society of Toxicology in Salt Lake City, UT in March, 2010.

CONCLUSIONS

Betulinic acid is a triterpenoid anticancer agent, and treatment of epidermal growth factor 2 (EGFR2, HER2 / ErbB2)-overexpressing BT474 and MDA-MB-453 cells with 1-10 μM BA inhibited cell growth and induced apoptosis. BA also induced proteasome-independent downregulation of specificity protein (Sp) transcription factors Sp1, Sp3, Sp4 and survivin, a Sp-regulated gene, and decreased expression of ErbB2, ErbB2-regulated kinases and YY1, a transcription factor that regulates ErbB2 expression in these cells. Knockdown of Sp1, Sp3, Sp4 and their combination by RNA interference was accompanied by decreased expression of ErbB2, YY1 and luciferase activity in cells transfected with a construct containing the GC-rich YY1 promoter linked to a luciferase reporter gene. BA-dependent repression of Sp1, Sp3, Sp4 and Sp regulated genes was due, in part, to induction of the Sp repressor ZBTB10 and downregulation of microRNA-27a (miR-27a) which constitutively inhibits ZBTB10 expression. The effects of BA on the miR-27a:zBTB10-Sp transcription factor axis were inhibited in cells cotreated with the cannabinoid 1 (CB1) and CB2 receptor antagonists AM251 and AM630 respectively. However, in vitro binding studies with ≤10 μM BA and a radiolabled cannabinoid did not indicate competitive binding of BA to the CB1 and CB2 receptors, suggesting a possible role for other CB-like G protein-coupled receptors.

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APPENDIX AND SUPPORTING DATA

Figure 1. Effects of BA on cell proliferation and apoptosis. BA-mediated inhibition of BT474 (A) and MDA-MB-453 (B) cell growth. Cells were treated with different concentrations of BA for up to 4 days and the number of cells in each treatment group was determined. Significant ($p<0.05$) growth inhibition is indicated (*). Results are expressed as means ± SE for at least 3 replicate determinations for each treatment group. (C) Effects of BA on cleaved-PARP and survivin. Cells were treated with 10 μM BA for 48 hr and whole cell lysates were analyzed by western blots. (D). BA induces apoptosis in cancer cells. Cells were treated with DMSO or 10 μM BA for 24 hr and analyzed with a TUNEL assay.

Figure 2. Effects of BA on Sp1, Sp3, Sp4, ErbB2 and ErbB2 dependent proteins. (A) BA decreases Sp protein and survivin levels in BT474 and MDA-MB-453 cells. Cells were treated with 10 μM BA alone or in combination with 1 μM lactacystin for 48 hr, and whole cell lysates were analyzed by western blots. (B) BA decreases mRNA levels of Sp proteins. Cells were treated with 10 μM BA for 16 hr, and mRNA levels were determined. Results are expressed as means ± SE for 3 replicate determinations for each treatment group and significant ($p<0.05$) decreases are indicated (*). (C) BA decreases protein levels of ErbB2 and ErbB2-dependent proteins. Cells were treated with 10 μM BA alone or in combination with 1 μM lactacystin for 48 hr, and whole cell lysates were analyzed by western blots. (D) BA decreases YY1 promoter activity. MDA-MB-453 cells were transfected with empty vector (PGL2), the YY1 p-277-luc or the YY1 p-1729-Luc construct, and cells were then treated with 5 or 10 μM BA for 24 hr. Luciferase activity was determined. Results are means ± SE for 3 separate determinations and significant ($p<0.05$) induction of luciferase activity by AP-2 is indicated (*).

Figure 3. Role of Sp proteins in regulating level of YY1 protein. Knock down of Sp1, Sp3, Sp4 or Sp1/Sp3/Sp4 in combination decreases protein levels of YY1 and ErbB2 in BT474 (A) and MDA-MB-453 (B) cells. Cells were transfected with siRNAs for Sp1, Sp3, Sp4 or Sp1/Sp3/Sp4 in combination for 72 hr, and whole cell lysates were analyzed by western blots. (C) Knock down of YY1 decreases protein levels of ErbB2 in BT474 and MDA-MB-453 cells. Cells were transfected with siRNA for YY1 for 72 hr, and whole cell lysates were analyzed by western blots.

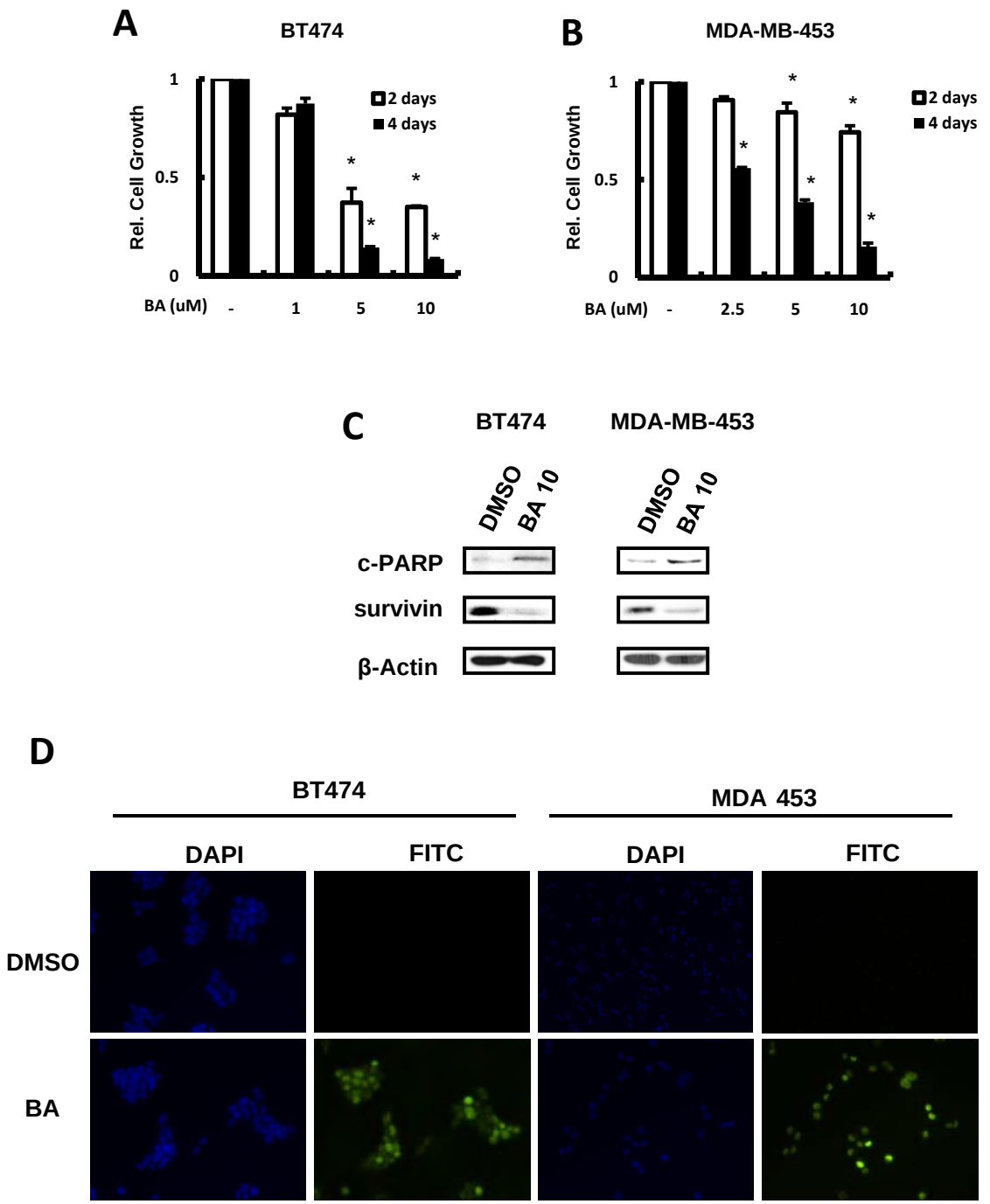
Figure 4. Role of cannabinoid receptors on effects of BA. Effects of AM251, AM630 and capsazepine on BA-mediated repression of Sps and survivin proteins in BT474 (A) and MDA-MB-453 (B) cells. Effects of AM251, AM630 and capsazepine on BA-mediated downregulation of ErbB2 and ErbB2-regulated kinases in BT474 (C) and MDA-MB-453 (D) cells and expression of CB receptors (D). Cells were pretreated with or without 6 μM AM251, 6 μM AM630 or 2 μM capsazepine for 1 hr, and then DMSO or 10 μM BA were added to the medium for 48 hr, and whole cell lysates were analyzed by western blots.

Figure 5. Effects of BA on miR-27a and ZBTB10, and the role of cannabinoid receptors on BA-mediated effects. Downregulation of miR-27a (A) and induction of ZBTB10 (B) mRNA levels with BA treatment in BT474 and MDA-MB-453 cells. Cells were pretreated with or without 6 μM AM251 or 6 μM AM630 for 1 hr, and then DMSO or 5 μM or 10 μM BA were added to the medium for 24 hr. Total RNA was extracted and

miR-27a and ZBTB10 RNA levels were determined. Results are expressed as means ± SE for 3 replicate determinations for each treatment group and significant ($p<0.05$) decreases or inductions are indicated (*). (C) Effects of ZBTB10 overexpression and antisense miR-27a on Sp protein levels, YY1 and ErbB2 proteins. Cells were transfected with 1 μg pCMV6-XL4-ZBTB10 plasmid or empty vector, 50 nM antisense miR-27a (as-miR-27a) or control, and whole cell lysates were analyzed by western blots. (D) Effects of miR-27a mimic or as-miR-27a on luciferase activity in ZBTB10 3'-UTR-luc construct transfected cells. MiR-27a mimic (50 nM) or as-miR-27a were transfected into BT474 and MDA-MB-453 cells, and a dual luciferase reporter assay was performed according to the manufacturer's instructions. Results are expressed as means ± SE for 3 replicate determinations for each treatment group and significant ($p<0.05$) decreases or inductions are indicated (*).

Figure 6. BA inhibits tumor (BT474 xenografts) growth. Inhibition of tumor size (A) and weight (B). Athymic nude mice bearing BT474 cells as xenografts were treated with BA (20 mg/kg/d) and tumor sizes and weights were determined. Significantly ($p<0.05$) decreased tumor sizes and weights are indicated (*). (C) BA decreases expression of Sp1, Sp3 and Sp4 proteins in tumors. Whole cell lysates from corn oil and BA-treated tumors were analyzed by western blot. (D) Immunostaining for ErbB2 and Sp1. Fixed tumor tissue from corn oil and BA-treated mice were stained with ErbB2 and Sp1 antibodies.

Figure 1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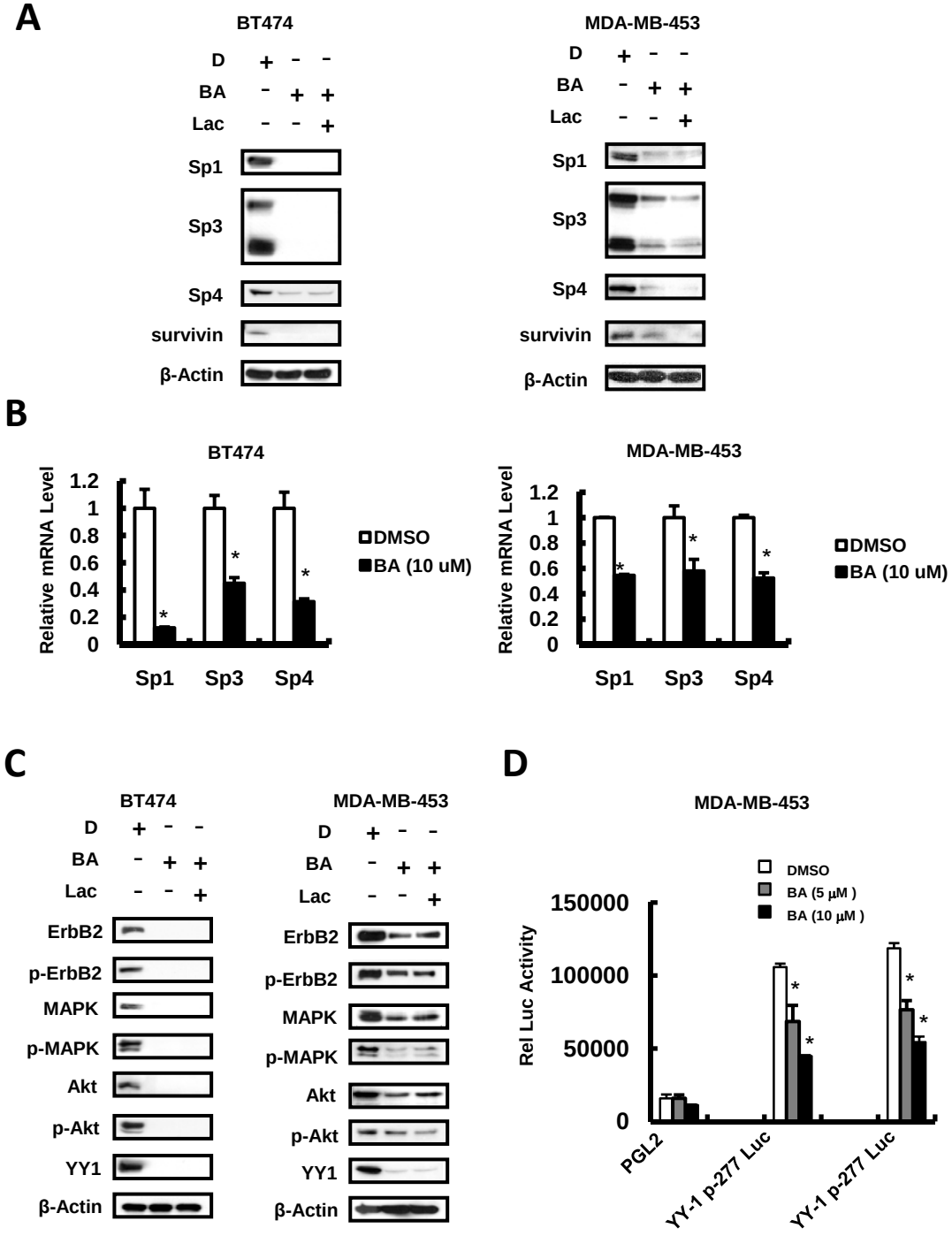
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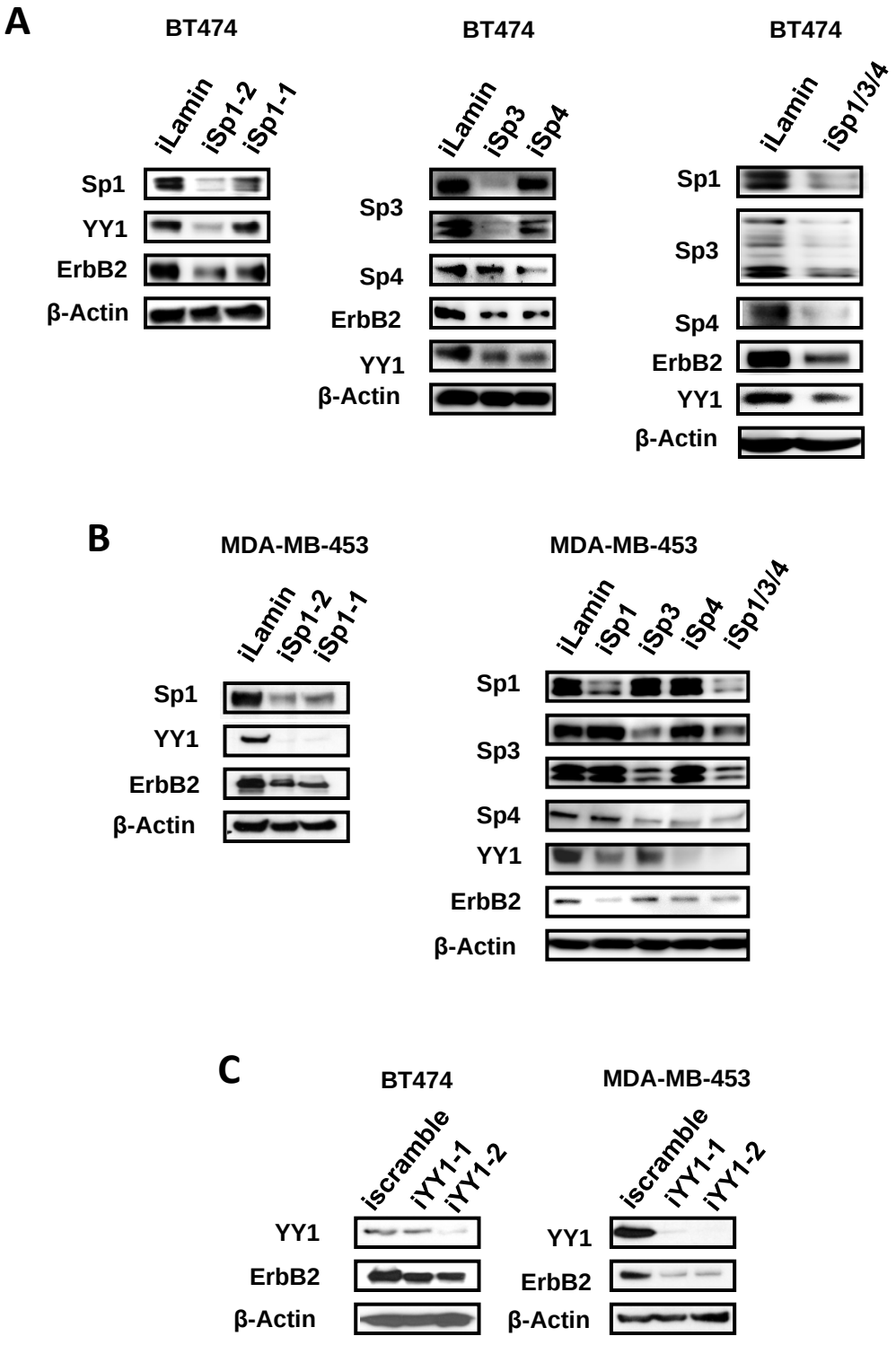
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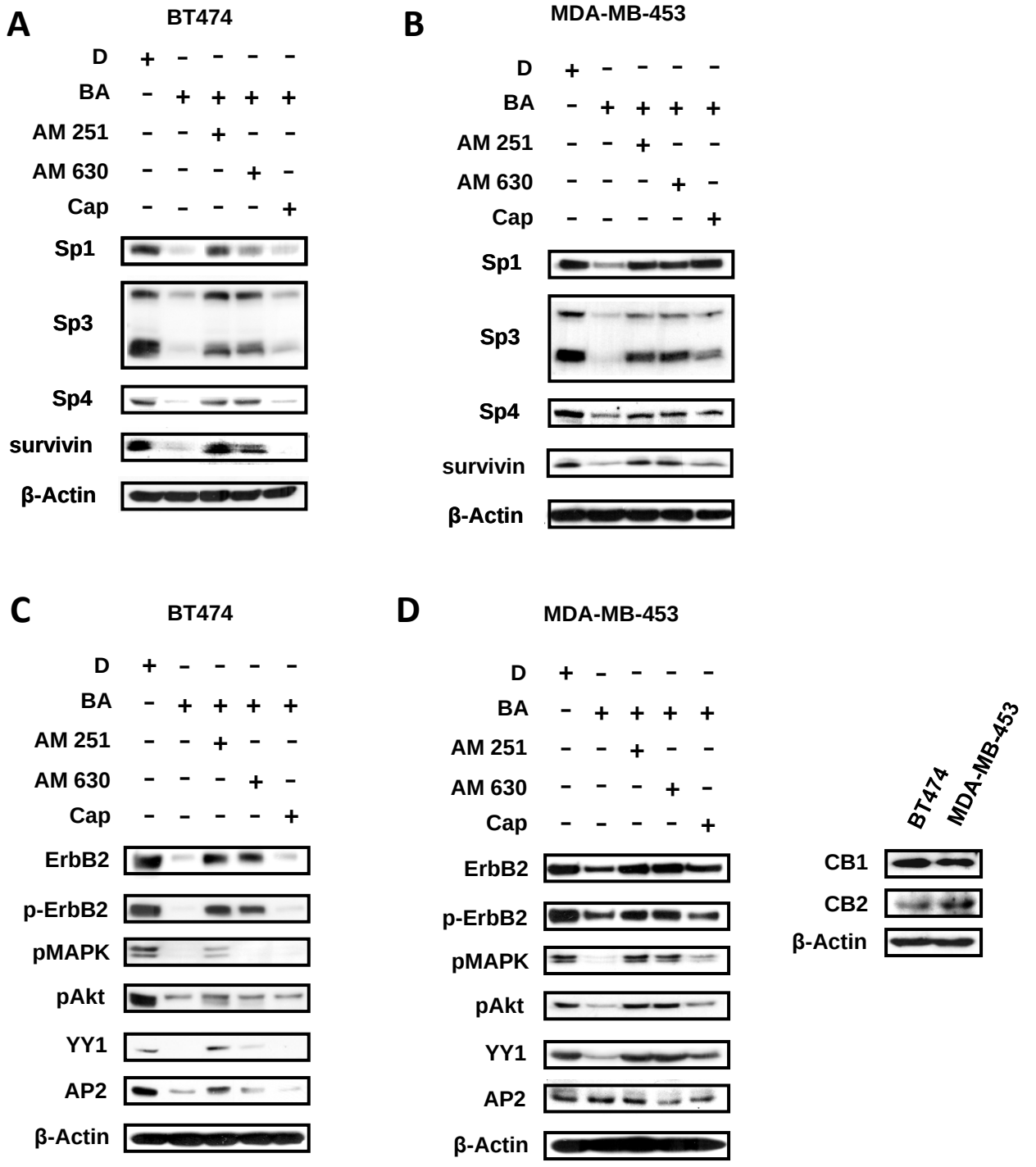
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BT474
D + – – – –
BA – + + + +
AM 251 – – + – –
AM 630 – – – + –
Cap – – – – +
Sp1
Sp3
Sp4
survivin
β-Actin
B
MDA-MB-453
D + – – – –
BA – + + + +
AM 251 – – + – –
AM 630 – – – + –
Cap – – – – +
Sp1
Sp3
Sp4
survivin
β-Actin
C
BT474
D + – – – –
BA – + + + +
AM 251 – – + – –
AM 630 – – – + –
Cap – – – – +
ErbB2
p-ErbB2
pMAPK
pAkt
YY1
AP2
β-Actin
D
MDA-MB-453
D + – – – –
BA – + + + +
AM 251 – – + – –
AM 630 – – – + –
Cap – – – – +
ErbB2
p-ErbB2
pMAPK
pAkt
YY1
AP2
β-Actin
BT474
MDA-MB-453
CB1
CB2
β-Actin

Figure 5

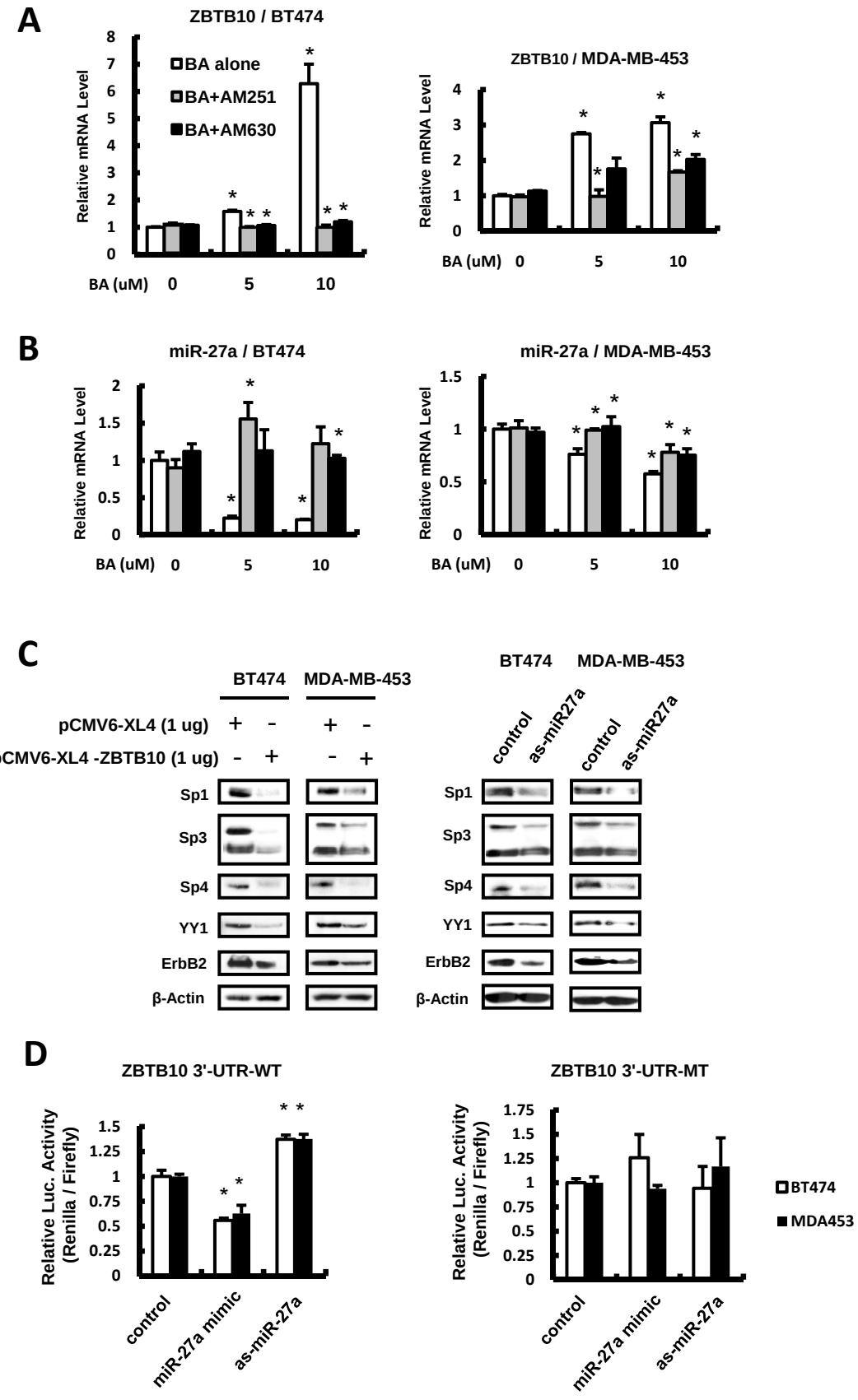
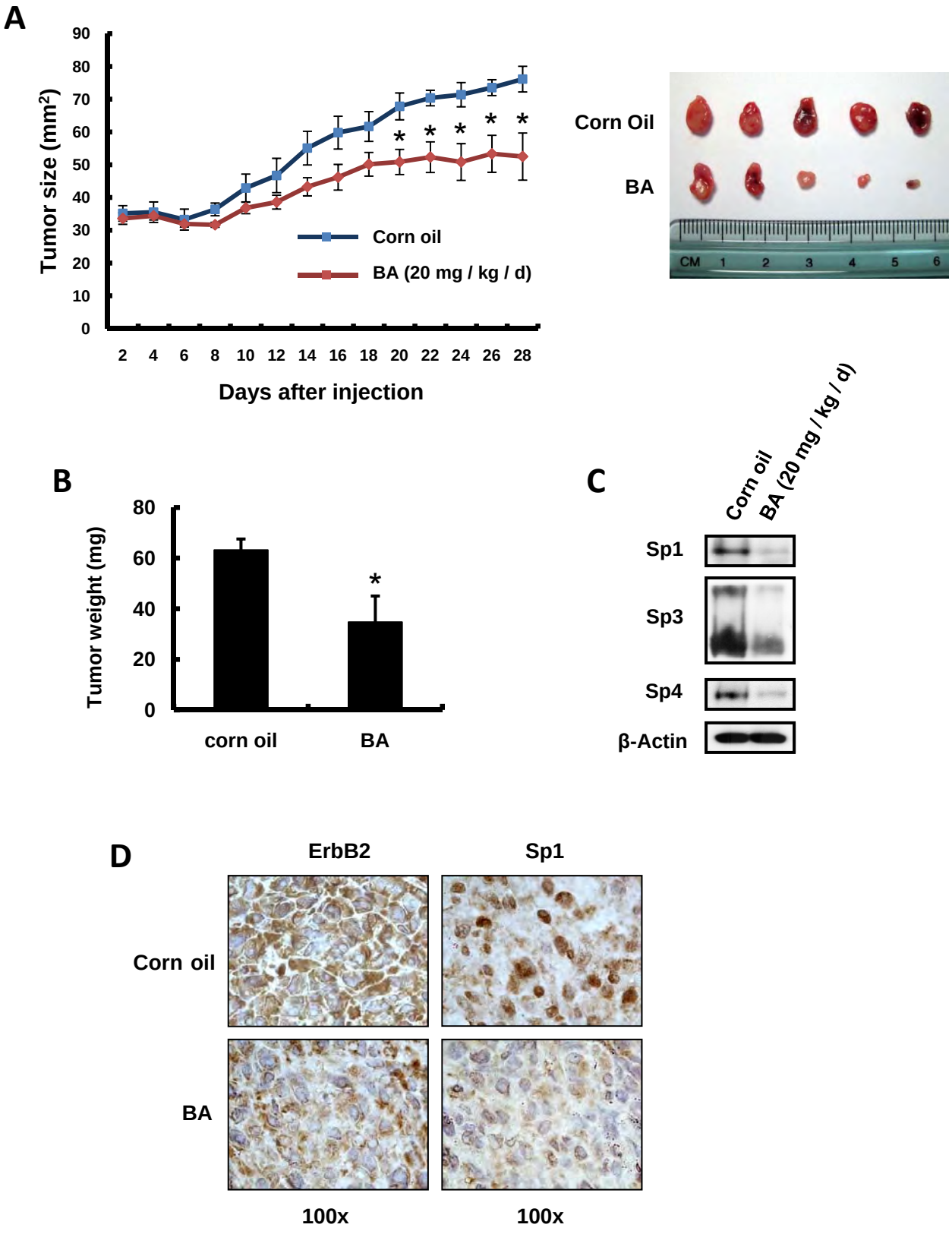

A
ZBTB10 / BT474
BA alone
BA+AM251
BA+AM630
Relative mRNA Level
BA (uM) 0 5 10
ZBTB10 / MDA-MB-453
B
miR-27a / BT474
miR-27a / MDA-MB-453
C
BT474
MDA-MB-453
pCMV6-XL4 (1 ug)
pCMV6-XL4 -ZBTB10 (1 ug)
Sp1
Sp3
Sp4
YY1
ErbB2
β-Actin
control
as-miR27a
D
ZBTB10 3'-UTR-WT
ZBTB10 3'-UTR-MT
Relative Luc. Activity (Renilla / Firefly)
control
miR-27a mimic
as-miR-27a
BT474
MDA453

Figure 6


A
Tumor size (mm²)
Days after injection
Corn oil
BA (20 mg / kg / d)
Corn Oil
BA
CM
B
Tumor weight (mg)
corn oil
BA
C
Corn oil
BA (20 mg / kg / d)
Sp1
Sp3
Sp4
β-Actin
D
ErbB2
Sp1
Corn oil
BA
100x
100x

Supplemental Figure 1